STUDIES ON

ALKALINE PHOSPHATASE

by

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Abstract

Food consumption in alloxan diabetic rats shows a positive correlation with serum alkaline phosphatase (SAP) activity, blood glucose level and body weight. Vitamin B_{12} given orally has no effect on SAP levels of normal or diabetic rats, nor does it modify the effect of supplementary methionine in either group, or of supplementary choline in normal rats. The addition of methionine to Purina Fox Checkers results in lowered SAP levels of both normal and diabetic rats due to the lowered consumption of this diet. Supplementary choline decreases SAP activity of normal rats but increases slightly the SAP levels in diabetic rats.

The effect of five inhibitors on the alkaline phosphatases of four tissues, in addition to serum, are presented and discussed. The evidence indicates that the increased SAP of alloxan diabetic rats, or of rats receiving a high-fat diet, originates in the intestine. The major portion of the SAP in normal rats appears to be derived from the intestine.

Fatty acids are the only dietary components which raise SAP activity above starvation levels. Oleic acid has a greater effect than stearic acid. Alkaline phosphatase appears to be involved in the intestinal absorption of saturated as well as unsaturated fatty acids.

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C O N T E N T S

I. INTRODUCTION

The term "alkaline phosphatase" is usually understood to refer to that enzyme which hydrolyzes monoesters of phosphoric acid at an optimal pH of approximately 9-10. Alkaline phosphatase activity is usually determined by measuring the rate of hydrolysis of sodium B-glycerophosphate or phenyl phosphate, although other substrates are also used. Magnesium is usually added to the incubation mixture, since the enzyme is activated by this ion in very dilute concentrations.

The first discovery of phosphatase activity dates back to 1907 when Suzuki, Yoshimura and Takaishi (1) found that certain cereals contained an enzyme which hydrolyzed phytin with the production of inorganic phosphoric acid. This enzyme is now known as phytase and is different from alkaline phosphatase. Since then, many different types of phosphatases have been discovered and investigated; and they have been found to occur in a great diversity of living tissues both plant and animal. For a comprehensive review and classification of the phosphatases the reader is referred to the review by Folley and Kay (2).

Alkaline phosphatase occurs in greatest concentrations in kidney, bone, intestine, mammary gland, lung, blood plasma, leucocytes and liver, although most

other mammalian tissues contain this enzyme. Recent studies (3 - 7) on the characteristics of the tissue alkaline phosphatases have led to the general conclusion that each tissue has a distinct phosphatase. Each tissue phosphatase differs from that of other tissues in behaviour toward inhibitors and activators, pH optima, and degree of reactivity with various substrates. This conclusion is not absolute, since some tissue phosphatases are almost indistinguishable, and the enzyme in some tissues undoubtedly is derived, in whole or in part, from outside sources.

The function of alkaline phosphatase in various tissues has been the subject of much investigation. The large concentration of alkaline phosphatase in bone, particularily of younger animals, led to studies on the role of phosphatase in calcification. Robison and his coworkers (8 - 10) investigated this problem and concluded that the enzyme acts on hexose monophosphate, releasing phosphate ion which affects the product of calcium and phosphate ion concentrations to such a degree that the solubility product is exceeded and calcium phosphate is precipitated. Neuman, Distefano and Mulryan (11) showed that ester phosphate inhibits calcification when phosphatase is absent, can form a complex with calcium ions, and that glycerol phosphate is used in bone

formation. The three-fold role of phosphatase in bone calcification is, then, to increase the concentration of free phosphate ion, to exert a protective action by preventing the adsorption of ester phosphate, and to increase the calcium concentration by destroying the complex forming agent. Zetterstrom (12, 13) has recently shown that bone phosphatase is activated as much as 400% by vitamin D_2 phosphate, and kidney phosphatase is activated 200%. This would explain the role of vitamin D in bone formation, and also the poor intestinal and renal absorption associated with deficiency of this vitamin. The problem of bone formation is still very controversial, however, as Siffert (14) concluded from histochemical studies that bone phosphatase is related to the elaboration of bone matrix, and that any function in making inorganic salts available is a secondary one.

The alkaline phosphatase of the kidney has been shown to have an important function in the resorption of glucose in the convoluted tubules. This function was first suggested by Lundsgaard (15) in 1933 and it has been substantiated since then (16 - 18). Glucose is phosphorylated during its passage through the convoluted tubules and is dephosphorylated during the resorption process. Phosphatase probably acts in the dephosphorylation stage, since the cells of the convoluted

tubules are very rich in the enzyme while the glomeruli, organs of filtration, do not contain phosphatase in most animal species.

Intestinal mucosa has the highest concentration of alkaline phosphatase of any of the tissues and many authors (19 - 23) consider that it may participate in the absorption of glucose and/or fatty acids. Lundsgaard (15) and Verzar (24, 25) support the hypothesis that glucose is phosphorylated and then dephosphorylated during absorption. Feeding of fat, on the other hand, has been shown to result in higher alkaline phosphatase levels of the intestine (26) and intestinal lymph (27) than the feeding of carbohydrates. The mechanism of phosphatase action in the intestine during the absorption of glucose and/or fatty acids is still largely unknown.

When glycogen in the liver is mobilized, it is broken down into glucose for transport by the blood. Hexose phosphates are intermediates in this transformation and alkaline phosphatase undoubtedly functions to hydrolyze glucose-6-phosphate to free glucose.

Serum alkaline phosphatase levels are used clinically in the diagnosis of certain diseases. Many bone diseases as well as obstructive jaundice result in elevated serum alkaline phosphatase levels in the human.

Satisfactory explanations for these phenomena are not available in some cases.

Workers in this laboratory have investigated factors affecting the serum alkaline phosphatase levels in rats. Cantor, Tuba and Capsey (28) found that rats with alloxan diabetes had abnormally high serum phosphatases which could be affected by alterations in the diets received by the animals. Tuba, Baker and Cantor (29) found a pronounced diurnal variation in the level of the enzyme, associated with the time of feeding, which necessitated bleeding the animals at 8 a.m. in order to standardize the effect of this factor. They showed that serum alkaline phosphatase activity varied directly with the daily food consumption, and that the influences of sex, castration and sex hormones upon the enzyme levels were exerted through the food consumption. Cantor, Wight and Tuba (30) proved that it was the fat content of the diet, rather than the caloric intake, that determined the level of the serum enzyme. Tuba, Cantor and Richards (31) found that weanling rats fed on a diet consisting chiefly of barley with a low protein content had greatly elevated serum alkaline phosphatases. When methionine was added to the diet the enzyme levels were reduced to nearly normal values.

Tuba and Shaw (32) studied the effect of diet-

ary fat, methionine and cystine on the serum phosphatase activity of weanling rats. The serum phosphatase levels increased in direct correlation to increased fat ingestion but addition of methionine to the diet reduced these high enzyme levels to nearly normal values.

This earlier work has now been extended and a study has been made of the effect of methionine and other lipotropic substances on the levels of serum alkaline phosphatase in relation to food consumption. The source of the alkaline phosphatase in serum was then investigated in order to explain the effect of the various factors mentioned above. Finally, a study of the function of the alkaline phosphatase in the intestine was begun.

II. METHODS

1. The Experimental Animal

Adult male albino rats were used in all experiments. They were housed individually in metal cages and were given their diets and tap water ad libitum. The individual daily food consumptions were recorded weekly. Body weights were taken whenever blood samples were collected, usually once per week.

Sampling of blood of the animals in the dietary experiments was accomplished by "milking" the blood from the tail into small tubes which were centrifuged within an hour after bleeding. The sera, separated from the formed elements, were used for the determination of alkaline phosphatase either immediately following separation or after having been stored at 5° C. for not more than two days. Blood specimens were obtained at approximately 9:00 a.m., unless otherwise noted.

Diabetes was induced in adult male albino rats by the subcutaneous injection of alloxan (Eastman Kodak) into rats which had been starved overnight. One ml. of a 3.2% solution of alloxan was injected per 200 gram rat. Blood sugars were determined on the blood of these animals one week after the alloxan injection and those rats with blood sugars 200% or more of normal were considered to be diabetic.

2. Phosphatase Determination

Alkaline phosphatases of the serum and other tissues were determined by the micromethod of Shinowara, Jones and Reinhart (33), as modified by Gould and Shwachman (34). This procedure measures the rate of hydrolysis of sodium B-glycerophosphate by phosphatase in terms of phosphate ion liberated in the reaction. The phosphate liberated is measured by the method of Kuttner and Lichtenstein (35). The unit of phosphatase activity is defined by Shinowara et al as "equivalent to one milligram of phosphorus liberated as phosphate ion during one hour of incubation at 37°C., with a substrate containing sodium B-glycerophosphate, hydrolysis not exceeding 10% of the substrate, and pH optimum of the reaction mixture for the alkaline enzyme at 9.3 ± 0.15".

(a) Reagents

(1) Substrate - buffer mixture

0.4240 gm. sodium diethyl barbiturate
0.5000 gm. sodium B-glycerophosphate
0.2464 gm. magnesium sulfate heptahydrate
dissolved in 100 ml. cold double distilled carbonate free water.

(2) Molybdic acid

Prepared daily by adding one part of a 7.5% sodium molybdate solution to one part of

cold ten normal sulfuric acid with constant shaking.

(3) Stannous chloride

I Stock solution - 6.0 gm. stannous chloride and 10 ml. hydrochloric acid. Covered with a toluene layer and stored in the refrigerator.

II Dilute solution - 0.2 ml. of stock solution diluted to 100 ml. with cold double distilled water. Kept cold and used within four hours.

(4) Stock phosphorus solution

0.4394 gm. potassium diphosphate made up to one litre. (100.1 micrograms of phosphorus per ml.)

(b) Method

Inorganic phosphorus was determined on 0.2 ml. of a 1:10 dilution of the serum or the supernatant from centrifuged tissue homogenates. Alkaline phosphatase was determined on 0.2 ml. of a 1:100 dilution of serum or a 1:10 dilution of supernatant from centrifuged tissue homogenates. Diluted sera were pipetted into two 10x75 mm. test tubes and 0.4 ml. of water was added to the serum being tested for inorganic phosphorus. The test tubes

and their contents were warmed on a water bath at 37°C., and then 0.4 ml. of the substrate-buffer mixture, also at 37°C., was added. Hydrolysis was allowed to proceed for precisely one hour and then 0.4 ml. of 10% trichloracetic acid was run into the tubes to precipitate the proteins and stop the enzyme action.

The tubes were centrifuged and the phosphorus concentrations were determined on 0.5 ml. portions of the supernatants, which were placed in 13x100 mm. test tubes. To each 0.5 ml. portion was added 0.7 ml. of 0.1 N NaOH and, in quick succession, with constant and vigorous shaking, 0.4 ml. of molybdic acid and 0.4 ml. of $SnCl_2$. All reagents added were cold. The intensity of the blue color which develops due to the formation of reduced oxides of molybdenum was read after 15 minutes in a Coleman Universal Spectrophotometer (Model 14) at 6000 A°, with a blank, consisting of 1.2 ml. of water, 0.4 ml. molybdic acid and 0.4 ml. $SnCl_2$, set at 100% transmission.

The percent transmission of each sample was converted into concentration of phosphorus by the use of a standard graph which was prepared by plotting the values of a number of different known values of phosphorus concentration (KH_2PO_4) against their percent transmission.

3. Blood Sugar Determination

Blood sugar was determined by the micromethod of Reinecke (36). Protein was precipitated with tungstic acid. The sugar in the centrifuged supernatant solution was oxidised with alkaline potassium ferricyanide. The ferrocyanide so formed was estimated colorimetrically, after conversion to Prussian Blue.

(a) Reagents

(1) Ferric iron gum ghatti solution

Gum ghatti tears were suspended for 18 hours in a copper screen at the top of a tall container filled with water (20 gm./ litre water). The solution was filtered and 7 gm. ferric sulfate hydrate dissolved in 75 ml. 85% phosphoric acid were added per litre. 1% $KMnO_4$ was added slowly until a trace of pink persisted for 15 minutes in order to destroy certain reducing substances present in the gum ghatti. The solution keeps indefinitely.

(2) Cyanide - carbonate buffer

4 gm. sodium carbonate dissolved in 25 ml. water were added to 0.75 gm. sodium cyanide dissolved in 75 ml. water and diluted to 500 ml.

(3) Potassium ferricyanide solution

250 mg. potassium ferricyanide in 500 ml. of water.

(4) Dilute tungstic acid

10 ml. of 0.67 N sulfuric acid and 10 ml. of 10% sodium tungstate were diluted to 500 ml.

(b) Method

A hemoglobin pipette was used to deliver 0.02 ml. of blood into 5 ml. of the tungstic acid reagent, and the pipette was rinsed several times with the acid. A stream of air bubbles blown by mouth through the pipette was used to mix the liquids. After 15 minutes the mixture was centrifuged and 1 ml. of the supernatant solution was transferred to a 15x125 mm. test tube. 1 ml. of potassium ferricyanide solution was then added to the sample and to 1 ml. of water used as a blank. The tubes were heated for 15 seconds in a boiling water bath and 1 ml. of the buffer was added. The tubes were then heated for 15 minutes in the boiling water bath with large marbles covering them to act as condensers. After cooling the tubes in ice water to 30°C., 1 ml. of the ferric iron gum ghatti solution was added and 1 ml. of water was then added to bring the volume up to 5 ml. Thorough mixing was necessary at each addition of reagents.

After 30 minutes the color produced was read in a Coleman Universal Spectrophotometer (Model 14) at 6400 A^{o}, with the blank set at 100% transmission. Sugar concentration was calculated by comparison of the readings with a calibration curve constructed by carrying out the above procedure on glucose solutions of known concentration.

III. THE RELATIONSHIP OF TOTAL FOOD CONSUMPTION, METHIONINE, CHOLINE AND VITAMIN B_{12} TO THE ALKALINE PHOSPHATASE IN RAT SERUM

1. Introduction

Tuba and Shaw (32) studied the effect of dietary fat, methionine and cystine on the serum alkaline phosphatase activity of weanling male albino rats. Variations in the concentrations of fat and methionine in the synthetic diets fed to growing rats produced marked alterations in the levels of the enzyme, as well as in the daily food consumption. Cantor, Wight and Tuba (30) found that the abnormally high serum alkaline phosphatase levels associated with alloxan diabetic adult rats could be influenced by alterations in the diets received by the animals. This section is concerned with the effect of supplementary methionine, already studied in weanling animals, on the serum alkaline phosphatase and food consumption of normal and alloxan diabetic adult male rats. Vitamin B_{12}, which is reported to have lipotropic and transmethylating effects (37), was used as a dietary supplement as well as the lipotrope, choline.

2. Experimental

Adult male albino rats, both alloxan diabetic and normal, were fed ad libitum a basal diet of ground Purina Fox Checkers or the basal diet supplemented as indicated below. Serum alkaline phosphatase and blood sugar levels were determined by the methods described above.

3. Results and Discussion

The Relationship Between Total Food Consumption and Serum Alkaline Phosphatase of Alloxan Diabetic Rats

Nine adult male rats which manifested well established alloxan diabetes were placed in individual cages and they were given ground Purina Fox Checkers and water ad libitum for 3 days. At the end of that time the daily food consumption, body weights, blood glucose and serum alkaline phosphatase levels were determined for each animal. They were then fed successive decrements of food as indicated in Table I for 3 day periods. At the end of each period, the animals were weighed and estimations were made of the blood glucose and serum phosphatase. The results are shown in Table I and are illustrated in Figure 1.

TABLE I

The Effect of Limiting Food Consumption (grams/day) on Serum Alkaline Phosphatase Levels (units/100 ml.); Blood Glucose (mg.%) and Body Weights (grams) of Diabetic Rats. (Means of 9 rats) Each food level was fed for three days before estimations were carried out.

Consumption	26 ± 1* (100)**	24 (93)	19 (73)	14 (54)	9 (35)	4 ± 0 (15)
Phosphatase	336 ± 17 (100)	354 (105)	247 (73)	177 (53)	135 (40)	61 ± 5 (18)
Blood Glucose	504 ± 44 (100)	429 (85)	295 (59)	262 (52)	244 (48)	135 ± 11 (27)
Body Weight	218 ± 9 (100)	213 (98)	195 (89)	186 (85)	173 (79)	146 ± 8 (67)

* Standard error of the mean.
** Figures in brackets are percentages of zero values.

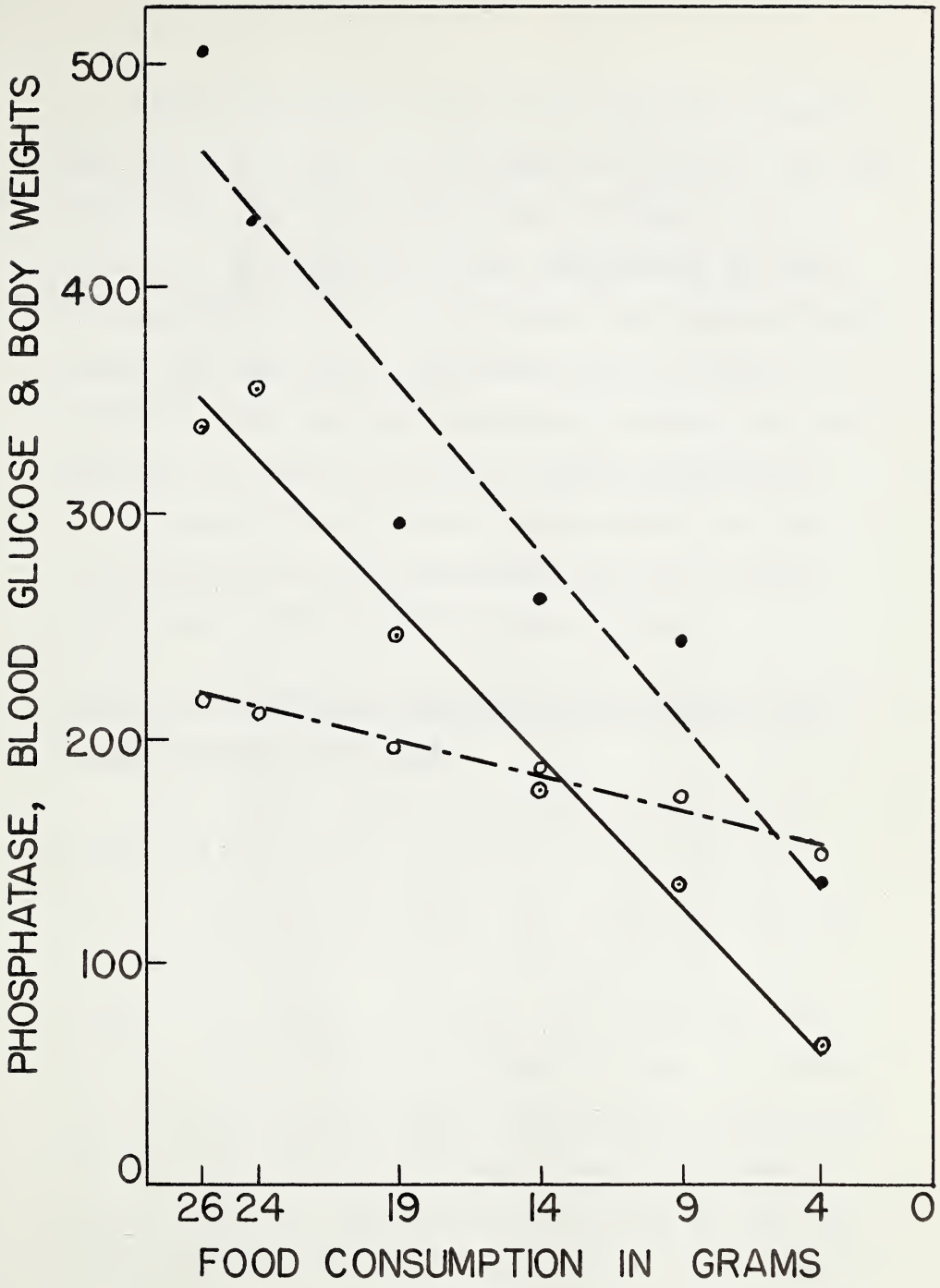

Fig. 1. The relationship between daily food consumption and serum alkaline phosphatase (———), blood glucose (— —), and body weights (— - —).

The correlation of mean serum alkaline phosphatase activity with food consumption is 0.99. The formula of the regression line shown in Figure 1 is $P = 13.27\ C + 6$, where P is the phosphatase activity in units/100 ml. and C is the daily food consumption in grams. The regression coefficient has a t value of 13.27 ($P < 0.01$) and the correlation between food consumption and enzyme activity is highly significant.

Correlations of blood glucose levels and of body weights with food consumption are also highly significant. (0.96 and 0.99 respectively.)

The Effect of Dietary Supplements Fed to Normal and Alloxan Diabetic Adult Rats

The effect on serum alkaline phosphatase of feeding various levels of food to normal adult male rats has been reported by Cantor, Wight and Tuba (30) and a highly significant correlation of 0.97 was obtained. Tuba, Baker and Cantor (29) found that variations in this serum enzyme occurred in male and female rats following gonadectomy or injection of various sex hormones, and that these changes could be related to food consumption. The correlation, which in this instance was 0.70, was significant, but it could also be concluded that other factors might exert a modifying influence.

The concentration of dietary fat appears to be very closely associated with levels of alkaline phosphatase in rat serum. Statistical analysis of the results of Cantor, Wight and Tuba (30) obtained with adult animals on limited, subnormal food intake, indicates a highly significant correlation of 0.86 ($P < 0.01$) between the daily intake of fat and the serum enzyme. The work of Tuba and Shaw (32) with weanling rats fed various synthetic diets ad libitum further confirms this relationship, and in this case the correlation is 0.98 ($P < 0.01$). It was noted from the results of Tuba and Shaw and from unpublished data from this laboratory that the influence of dietary fat may be modified by other factors in the diet. When methionine is used as a supplement, the usual correlation between food consumption and enzyme activity is not observed, or in other words, supplementary methionine in itself produces a significant alteration in the serum alkaline phosphatase levels of normal weanling rats.

The action of methionine in lowering serum alkaline phosphatase levels, presumably because of the lipotropic effect of the labile methyl groups, led to the use of this amino acid as a dietary supplement in normal and alloxan diabetic adult rats. Another lipo-

tropic substance, choline, was also used as a supplement as well as vitamin B_{12}, reported to have transmethylating and lipotropic actions (37). In all the following experiments a basal diet of ground Purina Fox Checkers was used.

The Effect of Vitamin B_{12}

Saline solutions of crystalline vitamin B_{12} were used. Oral supplements of this solution, equivalent to 1.0 microgram daily, were given to 8 normal and 7 alloxan diabetic adult rats for 3 days. The results, not reported here, showed that supplementary vitamin B_{12} had no significant effect on serum alkaline phosphatase levels of normal or alloxan diabetic animals.

The Effect of Methionine Alone or With Vitamin B_{12}

(a) Normal Rats

A group of 10 adult male rats was fed ad libitum a diet of ground checkers to which methionine had been added to a concentration of 3%. Another group of 10 animals received the same diet but in addition was given oral supplements of 1.0 microgram of vitamin B_{12} daily after the eighth day. The two groups were maintained on their respective diets for 17 days. The results in Table II give a correlation of serum

alkaline phosphatase activity with food intake of 0.92 ($P < 0.01$) for the group receiving supplementary methionine and of 0.90 for the group receiving supplements of methionine and vitamin B_{12}. Therefore in normal adult male rats the decrease in levels of the enzyme associated with methionine supplementation of the basal diet can be related to the lowered food consumption. This is contrary to the findings with weanling animals reported by Tuba and Shaw. Vitamin B_{12} produced no significant effect on the enzyme in addition to that attributable to methionine.

TABLE II

The Effect of Supplements of Methionine alone and Methionine with Vitamin B_{12} on Serum Alkaline Phosphatase (units/100 ml.) (P), Daily Food Consumption (grams) (C) and Body Weights (grams) (W) of normal rats. (Means of 10 rats) Vitamin B_{12} supplementation was begun on the 8th day.

Time	Methionine			Methionine + B_{12}		
	P	C	W	P	C	W
0 Day	119±6* (100)**	15.6±0.8 (100)	239±9 (100)	113±7 (100)	16.6±0.4 (100)	235±12 (100)
3 Days	90 (76)	13.1 (84)	234 (98)	79 (70)	12.8 (77)	228 (97)
7 Days	98 (83)	12.3 (79)	235 (98)	88 (78)	11.9 (72)	229 (98)
9 Days	81 (68)	11.2 (72)	231 (97)	69 (61)	10.5 (63)	225 (96)
14 Days	102 (86)	14.3 (92)	237 (99)	88 (78)	14.4 (87)	229 (98)
17 Days	106±7 (89)	15.0±0.2 (96)	242±8 (101)	94±4 (83)	15.3±0.1 (92)	235±3 (100)

* Standard error of the mean.
**Figures in brackets are percentages of zero values.

(b) Alloxan Diabetic Rats

The preceding experiment was repeated with alloxan diabetic adult male rats for twenty-one days. The results in Table III give a correlation of 0.99 ($P < 0.01$) between enzyme activity and food intake in the group receiving methionine and of 0.96 for the group receiving supplementary methionine and vitamin B_{12}. Therefore, in neither group did the supplementation of the diet affect the serum alkaline phosphatase levels, as in the experiment above with normal adult animals.

In all four groups of adult animals receiving supplementary methionine, after an initial adjustment to the dietary regimen, weights remained constant in spite of decreased food consumption. In previous experiments with weanling rats fed moderate supplements of methionine the rate of growth was similar to that of the unsupplemented animals, although food consumptions were appreciably lower (32). It would appear that supplementation with methionine results in nutritionally adequate diets which are characterized by lowered food consumption.

TABLE III

The Effect of Supplements of Methionine alone and Methionine with Vitamin B_{12} on Serum Alkaline Phosphatase (units/100 ml.) (P), Daily Food Consumption (grams) (C), Body Weights (grams) (W) and Blood Glucose (mg.%) (G) of diabetic rats. (Means of 10 animals) Vitamin B_{12} supplementation was begun on the 8th day. Control group returned to Checkers on 21st day.

Time	Methionine				Methionine + B_{12}			
	P	C	W	G	P	C	W	G
0 Day	307±20* (100)**	27.5±0.3 (100)	226±7 (100)	532±32 (100)	334±9 (100)	26.6±1.6 (100)	206±6 (100)	531±19 (100)
7 Days	213 (69)	19.5 (71)	210 (93)		217 (65)	19.6 (74)	184 (89)	
9 Days	219 (71)	19.1 (70)	210 (93)		235 (70)	18.7 (70)	185 (90)	
14 Days	209 (68)	18.3 (67)	210 (93)		235 (70)	18.7 (70)	189 (92)	
21 Days	206±14 (67)	18.7±0.7 (68)	211±16 (93)	348±12 (65)	231±10 (69)	19.1±1.8 (72)	190±8 (92)	376±16 (71)
29 Days***	271±16 (88)	24.6±2.0 (89)	235±15 (104)	454±21 (86)				

* Standard error of the mean.
** Figures in brackets are percentages of zero values.
*** 8 days back on stock diet.

The Effect of Choline Alone or With Vitamin B_{12}

(a) Normal Rats

Ten adult male rats were fed a diet consisting of the basal diet containing a supplement of 1.5% choline, and another group of 10 normal animals received the same diet and in addition oral supplements of 1.0 microgram of vitamin B_{12} each daily after the eighth day. Statistical analysis of the results in Table IV shows that there is no correlation between food consumption and phosphatase activity in either group. The results further indicate that supplementary choline produced a lowering of about 20% in the alkaline phosphatase activities of both groups. Comparison of mean phosphatase levels at the beginning of the experiment and at its termination 14 days later shows a significant decrease with a t value of 6.05 ($P < 0.01$) in the first group. During the experiment the animals gained in weight, which, in my experience, serves to emphasize the importance of the decrease in enzyme activity. Comparison of the final phosphatase values for the two groups shows that vitamin B_{12} had in itself no effect on the enzyme.

TABLE IV

The Effect of Supplements of Choline alone and Choline with Vitamin B_{12} on Serum Alkaline Phosphatase (units/100 ml.), Daily Food Consumption (grams) (C) and Body Weights (grams) (W) of normal rats. (Means of 10 rats) Vitamin B_{12} supplementation was begun on the 8th day.

Time	Choline			Choline + B_{12}		
	P	C	W	P	C	W
0 Day	150±6* (100)**	18.2±0.3 (100)	224±5 (100)	130±7 (100)	19.2±0.4 (100)	224±5 (100)
3 Days	137 (91)	17.7 (98)	229 (102)	113 (87)	17.9 (93)	231 (103)
7 Days	129 (86)	16.6 (91)	240 (107)	118 (91)	17.2 (90)	242 (108)
9 Days	118 (78)	15.1 (83)	239 (107)	109 (84)	15.9 (83)	243 (109)
14 Days	118±7 (78)	17.0±0.4 (94)	252±20 (113)	106±7 (82)	17.7±0.3 (92)	248±6 (111)

* Standard error of the mean.
**Figures in brackets are percentages of zero values.

(b) Alloxan Diabetic Rats

Ten animals which had been diabetic for three weeks were used. This period ensured the use of animals still in relatively good condition, and with serum alkaline phosphatase activity levelled off at about 200 - 300% above normal (30). They were fed the basal diet containing a supplement of 1.5% choline for 6 weeks, and at the end of that time the amount of added choline was increased to 2.5% for 7 additional days. A slight but statistically significant increase was found in the phosphatase values, which showed no correlation with food consumption. A rise of similar magnitude was noted in an identical experiment.

The effect of supplementary choline on the abnormally elevated alkaline phosphatases of the sera of alloxan diabetic rats is the opposite of the findings with normal rats. This finding further emphasizes the metabolic abnormalities found in this type of experimental diabetes.

The effect of oral vitamin B_{12} on the serum phosphatase activity of alloxan diabetic animals receiving supplementary choline was not investigated, in view of the lack of effect of the vitamin in the experiments above.

TABLE V

The Effect of Choline Supplementation on Serum Alkaline Phosphatase (units/100 ml.) (P), Daily Food Consumption (grams) (C) and Body Weights (grams) (W) of Diabetic Rats. (Means of 10 rats) The choline supplement was increased to 2.5% at the end of 6 weeks.

Time	P	C	W
0 Values	302±10* (100)**	27.3±0.9 (100)	212±6 (100)
1 Week	350 (116)	28.1 (103)	207 (98)
2 Weeks	328 (109)	30.4 (111)	208 (98)
3 Weeks	335 (111)	27.8 (102)	206 (97)
4 Weeks	328 (109)	27.7 (101)	204 (96)
5 Weeks	331 (110)	26.3 (97)	204 (96)
6 Weeks	332±6 (110)	26.8±1.2 (98)	203±7 (96)
1 Day on 2.5% Choline	302 (100)	23.8 (87)	199 (94)
3 Days on 2.5% Choline	332 (110)	25.7 (94)	200 (95)
7 Days on 2.5% Choline	340±7 (113)	24.2±0.4 (89)	205±8 (97)

* Standard error of the mean.
** Figures in brackets are percentages of zero values.

4. Summary

Food consumption in alloxan diabetic rats shows a positive correlation with (1) alkaline phosphatase activity of the serum, (2) blood glucose level, (3) body weight, similar to the correlation between enzyme activity and food intake in normal adult rats when fed a stock laboratory diet.

Oral vitamin B_{12} has no effect on serum alkaline phosphatase levels of normal or diabetic rats, nor does it modify the effect of supplementary methionine in either group. The effect of supplementary choline on the phosphatase of normal rats is unaltered by this vitamin, which is reported to have transmethylating and lipotropic influences.

Methionine added to Purina Fox Checkers lowers phosphatase levels of the serum of both normal and diabetic adult animals, but this is directly related to lowered food consumption which always accompanies supplementation with this amino acid. The animals maintain their weights and appear to be in good condition, so it may be assumed that the lowered intake of food is adequate.

Supplementary choline decreases serum phosphatase levels of normal rats by about 20% of initial values, and this is due to the choline itself and not to

altered food consumption. Phosphatase levels of alloxan diabetic rats, however, are slightly but significantly elevated by supplementary choline.

IV. ON THE SOURCE OF THE ALKALINE PHOSPHATASE IN RAT SERUM

I. Introduction

The results of the previous section confirm the findings of other workers (29, 30, 38) that serum alkaline phosphatase activity varies with food consumption; and that most factors affecting levels of the serum enzyme transmit their effect through alterations in food consumption. In attempting to explain these facts, it appeared logical to investigate the origin of serum phosphatase.

Alkaline phosphatase occurs in several tissues and consequently the source of the enzyme in serum has stimulated considerable investigation and speculation. Dalgaard (39) reported that the removal of the small intestine or the kidney from the dog does not prevent the increase in serum alkaline phosphatase activity which always follows ligation of the common bile duct in this animal. Freeman (40) concluded that the liver in the dog is the source of increased serum phosphatase because increased levels of the enzyme were greater following bile duct obstruction than following hepatectomy. There was a three-fold increase in serum alkaline phosphatase in the latter case, despite the fact that the animals died within two days, possibly without eating.

It is well known that obstructive jaundice in the human causes increased serum alkaline phosphatase activity.

Ligation of the common bile duct in the rat, however, was shown by Weil and Russel (41) to result in a sharp decrease in serum alkaline phosphatase levels, which could be prevented by the addition of bile to the diet. The low enzyme levels returned to normal as new bile ducts were formed. Other workers (42) reported that ligation of the common bile duct prevented the increase in alkaline phosphatase activity which normally accompanies a meal rich in fat. However, there was an increase in the plasma over fasting levels of the enzyme, although activity was still far below normal. These investigations with the rat would seem to indicate that the response of serum alkaline phosphatase to bile duct obstruction in this animal differs markedly from the response in the dog and in the human.

Weil and Russel (38), and, subsequently, Cantor, Wight and Tuba (30), found that the starving of normal rats reduced their serum alkaline phosphatase levels to very low values within 24 hours. The activity of the enzyme remained at constant, low levels throughout further starvation, but it was rapidly restored to normal when the animals were fed their stock laboratory diet. The former workers found that only the fat portion of

the diet was effective in raising fasting levels of the serum enzyme to normal. Gould (43) also showed that the feeding of fat to rats produced a marked increase in serum phosphatase activity. In addition, he observed that the inhibition of the serum enzyme by sodium taurocholate resembled the inhibition by this substance of intestinal rather than bone or kidney phosphatase.

Various workers in this laboratory (29, 30, 32) have obtained a highly significant correlation between the daily food consumption of rats and their serum phosphatase levels, and they have demonstrated that this correlation is associated with the amount of fat ingested. Laurie and Judkins (26), when studying the effect of diet upon rat intestinal phosphatase activity, found that diets containing 55% to 70% sucrose produced a lower concentration of the enzyme in the intestine than when the sucrose was replaced by fat or protein. Kosman, Karlberg and Freeman (44) found that the intestine of dogs secreted the greatest amount of phosphatase after a fat or protein meal, and least after ingestion of carbohydrates. A. Bodansky (20), however, reported that only carbohydrate would restore the low fasting levels of plasma alkaline phosphatase to their normal values in young dogs. He concluded that plasma alkaline phosphatase is of mixed origin, and that the intestine is an

important source. Furthermore, levels of the serum enzyme in various diseases reflect alterations in alkaline phosphatase concentration in the organs affected. The latter view is substantiated by Motzok (45), who showed that the elevated plasma phosphatase in the rachitic chick originates in the bone.

It would appear from the investigations referred to in the preceding paragraph that similar dietary regimens have similar effects upon the phosphatase levels of both the intestine and serum of various animals. The relationship between the enzyme in the intestine and serum was further elucidated by the studies of Flock and Bollman (27) on intestinal lymph. The phosphatase levels of the lymph were higher than the plasma levels, and these were decreased upon starving and increased again upon feeding. The changes in the lymph were paralleled by the changes in the plasma. The increases were greatest after a meal rich in fat. It was further observed that when all the lymph was collected continuously and not allowed to reach the blood, the plasma phosphatase was reduced to very low levels within 35 hours. They concluded that alkaline phosphatase is supplied to the blood from the small intestine by way of the lymph.

Several investigators have presented evidence to

support the hypothesis that the liver is an important source of serum alkaline phosphatase (46, 47), while others (39, 48) have concluded that the liver serves primarily as a regulator of the secretion of phosphatase into the bile. Gould and Schwachman (49) found that the decreased level of serum alkaline phosphatase in the scorbutic guinea pig was paralleled by decreased bone phosphatase activity. The inhibitory effect of sodium taurocholate on the serum phosphatase activity resembled the effect on bone and kidney phosphatase rather than on intestinal phosphatase. Armstrong and Banting (50) extirpated the intestines, kidneys, spleen, pancreas, liver, testes and epididymes of the dog without lowering the serum alkaline phosphatase activity, and they concluded that bone is the sole source.

There are numerous other papers concerned with the possible source of serum alkaline phosphatase. Unfortunately, many of them fail to consider the highly significant correlation which exists between food consumption and the phosphatase levels of both the serum and the intestine.

Certain inhibitors have been used to differentiate the alkaline phosphatases of the tissues. Cloetens (4) separated the phosphatases of liver extract

into a cyanide sensitive and a cyanide insensitive fraction. Various workers have used cyanide inhibition of certain tissue alkaline phosphatases in an attempt to elucidate the source of the serum enzyme (51-53). Oxalate will also inhibit the phosphatases of different tissues to varying degrees (54). O. Bodansky (55) found that bile acids inhibited bone and kidney phosphatases but that they had no effect on intestinal phosphatase. He also studied the inhibition of tissue phosphatases by amino acids (3). Glycine and D-L-alanine inhibited the phosphatases of bone, kidney and intestine to the same extent, while L-glutamic acid inhibited intestinal phosphatase more than it did bone or kidney phosphatases. L-lysine and L-histidine caused a lesser degree of inhibition of intestinal phosphatase than of the other two tissues.

The evidence so far presented by various workers on the origin of rat serum alkaline phosphatase tends to be inconclusive. Work in this laboratory has clearly shown that a number of factors (28 - 32) markedly influence the levels of rat serum alkaline phosphatase, and it seemed possible that some of these would be useful in attempting to solve the problem insofar as the rat is concerned. In addition, the successful employment of inhibitors to differentiate the phosphatases

of various tissues led to the use of some of these in this investigation. The effects of five inhibitors upon the alkaline phosphatases of four tissues, in addition to serum, were studied simultaneously under identical experimental conditions in order to obtain a comprehensive view. The mechanism of inhibition by any of these substances is not considered in this thesis.

2. Experimental

Adult male albino rats were used in all experiments. They were killed by exsanguination, and the serum, which was separated immediately, was stored in the refrigerator. The liver, one kidney, the first ten cm. of the small intestine from the pylorus, and the femur and tibia of one leg were removed at once and placed on ice until they could be prepared. All tissues were cleaned of extraneous material, washed with cold distilled water, blotted dry, and weighed. They were then homogenized with approximately 100 ml. of cold distilled water for four minutes in a Waring blendor. The homogenates were placed in 200 ml. volumetric flasks (250 ml. for intestine) and stored overnight at 5°C. In the morning the homogenates were made up to volume and shaken, and a portion of each was then centrifuged. An aliquot of the supernatant thus obtained was diluted ten times and used at once in the experiments.

Phosphatase activity was determined by the micromethod described in Section II. pH optimum tests were run on the tissues and while there were slight differences it was felt that these were not sufficient to prevent doing all the tests at the same pH of 9.3. The inhibitors used were sodium taurocholate, sodium cyanide, sodium oxalate, L-glutamic acid and L-lysine.

These were included in the usual substrate - buffer mixture and the pH was adjusted to give a pH in the incubation mixture of 9.3 $\pm$ 0.15, in order to obviate inhibition which could be attributed to changes in hydrogen-ion concentration. The concentrations of inhibitors reported in the tables are for the incubation mixtures.

2. Results

Alloxan Diabetic Rats

Cantor, Tuba and Capsey (28) reported that rats with alloxan diabetes had greatly increased serum alkaline phosphatases and an attempt was made to ascertain, if possible, the source of this extra phosphatase. A group of animals manifesting well established alloxan diabetes, with blood sugars approximately 400% of normal, were used in this experiment. They were given ground Purina Fox Checkers and water ad libitum. The results are reported in Table VI and are illustrated in Figure 2. See discussion on pages 51 to 54.

Rats Receiving 25% Fat Diet

It was next attempted to determine whether the increased serum alkaline phosphatase caused by the feeding of fat to rats (32, 43) is of the same nature and source as that produced by alloxan diabetes. Rats for this experiment were starved for three days and then fed a diet containing 25% fat for two days. This resulted in serum alkaline phosphatase levels which were much higher than normal. The diet used consisted of 23% hydrogenated vegetable oils (Crisco), 2% cod liver oil, 20% casein, 51% sucrose, 4% McCollum's Salt Mixture, together with adequate supplements of the

B-group vitamins. The following amounts of the B-group vitamins were added to each kilogram of diet:

thiamine hydrochloride..... 5 mg.
calcium pantothenate.....20 mg.
pyridoxine hydrochloride..... 5 mg.
niacin..... 5 mg.
riboflavin.....10 mg.
choline..... 1 gram.

The results are reported in Table VII and are illustrated in Figure 3.

Normal Rats

Rats maintained on an ad libitum diet of Purina Fox Checkers were used. The results are given in Table VIII and are illustrated in Figure 4.

Starved Rats

Rats previously fed on checkers were starved for one week before being used in the experiments. The results are shown in Table IX and are illustrated in Figure 5.

TABLE VI

The Effect of Inhibitors on the Tissue Alkaline Phosphatase Activities of Alloxan Diabetic Rats

Each value is the mean of 4 rats

Tissues		Kidney	Liver	Intestine	Bone	Serum
Original Values of Alkaline Phosphatase in Units per 100 g. Wet Tissue: Serum, /100 ml.		3,490±390*	248±19	14,200±700	1,210±90	320±26
Inhibitor	**Molar Conc.**	**Residual Phosphatase Activity as Percentages of Original Values**				
Sodium Taurocholate	0.001	76.3±2.0*	93.4±2.1	101±2.5	87.7±1.5	101±0.2
Sodium Cyanide	0.01	11.9±1.6	46.8±1.6	5.0±0.6	11.4±2.2	4.6±0.9
	0.001	30.0±1.9	54.3±3.2	6.5±0.2	23.4±6.1	10.2±1.6
Sodium Oxalate	0.1	23.4±4.0	0	19.7±3.5	13.4±5.6	21.7±1.0
	0.01	84.7±8.4	77.4±5.1	86.9±4.3	93.4±6.4	87.2±2.6
L-(+)-Glutamic Acid	0.05	76.4±2.2	69.2±1.9	17.0±1.1	62.2±4.2	14.6±1.5
	0.01	106.2±2.4	77.0±2.3	53.8±2.1	93.4±13	57.7±1.1
L-(+)-Lysine	0.1	18.9±1.4	41.2±0.5	11.0±1.0	21.0±3.0	12.1±0.3
	0.01	70.3±2.4	72.1±1.2	59.4±4.4	68.7±3.0	67.3±6.7

*Standard error of the mean.

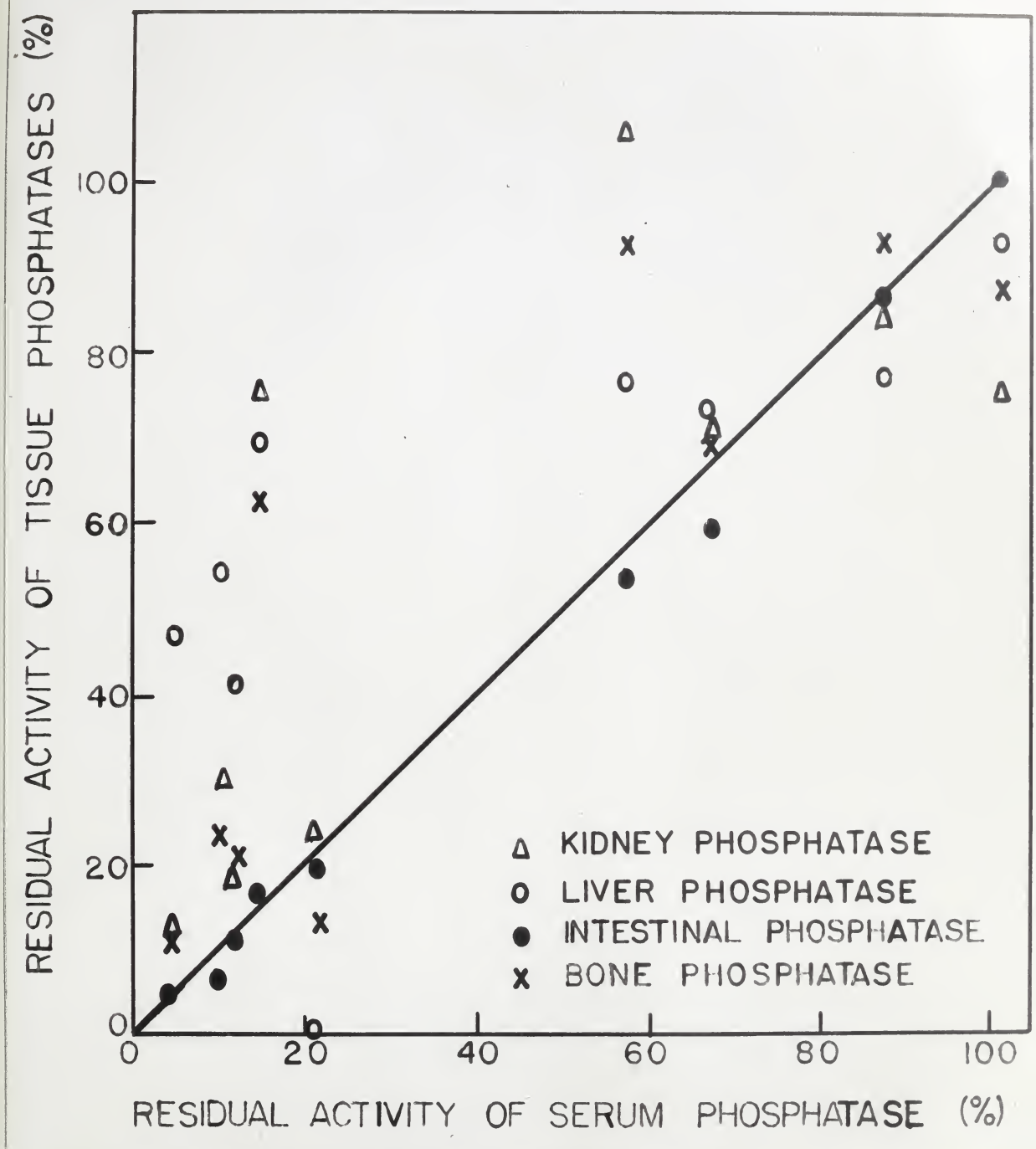

Fig. 2. Residual activity of tissue alkaline phosphatases in the presence of various inhibitors plotted against the corresponding residual phosphatase of serum in DIABETIC adult rats. Each point represents the mean value with one inhibitor for 4 rats. The line drawn through the origin with a slope of 45° represents the theoretical graph of any tissue phosphatase which manifests a pattern of behaviour towars the inhibitors identical with that of serum alkaline phosphatase.

TABLE VII

The Effect of Inhibitors on the Tissue Alkaline Phosphatase Activities of Rats on a 25% Fat Diet

Each value is the mean of 5 rats

Tissues		Kidney	Liver	Intestine	Bone	Serum
Original Values of Alkaline Phosphatase in Units/100 g. Wet Tissue: Serum, /100 ml.		3,520±160*	172±5	13,500±2,200	1,280±100	175±26
Inhibitor	**Molar Conc.**	**Residual Phosphatase Activity as Percentages of Original Values**				
Sodium Taurocholate	0.001	72.2±3.4*	84.4±3.7	102.4±10	85.4±5.4	100.6±0.7
Sodium Cyanide	0.01	13.6±0.9	47.1±4.4	8.2±1.4	12.5±1.6	8.9±1.8
	0.001	32.9±2.4	59.2±4.4	9.6±1.5	31.0±4.0	9.3±2.0
Sodium Oxalate	0.1	20.3±1.7	0	12.5±3.8	17.1±1.5	18.7±1.4
	0.01	84.2±4.8	72.6±1.9	86.8±5.1	81.5±9.9	84.8±1.0
L-(+)-Glutamic Acid	0.05	78.8±1.3	74.6±3.1	17.0±1.6	75.9±5.4	17.9±1.4
	0.01	111.0±5.1	91.6±5.1	60.9±3.3	118.4±8.7	59.8±2.4
L-(+)-Lysine	0.1	22.5±1.7	44.7±3.6	15.9±2.1	27.9±3.2	14.6±2.5
	0.01	68.3±3.1	74.3±1.4	70.2±4.6	74.2±4.8	65.2±3.5

*Standard error of the mean.

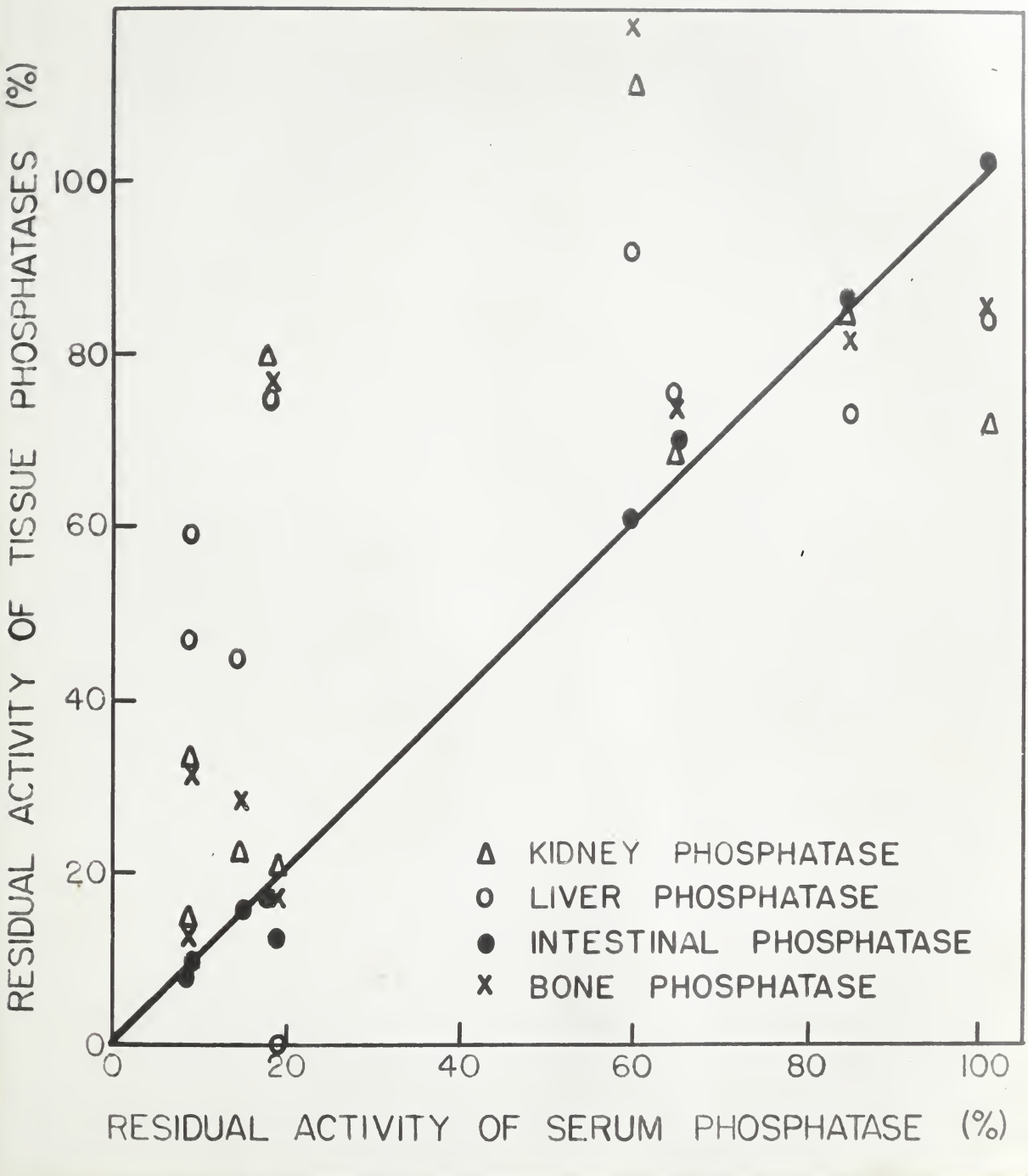

Fig. 3. Residual activity of tissue alkaline phosphatases in the presence of various inhibitors plotted against the corresponding residual phosphatase activity of serum in adult rats receiving a HIGH FAT DIET. Each point represents the mean value with one inhibitor for 5 rats. See legend to Fig. 2.

TABLE VIII

The Effect of Inhibitors on the Tissue Alkaline Phosphatase Activities of Normal Rats

Each value is the mean of 6 rats

Tissues		Kidney	Liver	Intestine	Bone	Serum
Original Values of Alkaline Phosphatase in Units/100 g. Wet Tissue: Serum, /100 ml.		4,350±230*	147±7	8,400±1,400	1,070±70	90±12
Inhibitor	Molar Conc.	Residual Phosphatase Activity as Percentages of Original Values				
Sodium Taurocholate	0.001	72.8±3.6*	94.6±2.1	113.6±2.3	85.8±3.5	96.6±2.1
Sodium Cyanide	0.01	9.8±1.5	52.8±3.1	7.4±1.9	14.4±0.9	9.5±2.1
	0.001	29.0±1.9	66.0±0.8	8.8±1.3	32.1±2.6	17.1±2.2
Sodium Oxalate	0.1	21.2±2.2	0	17.7±1.8	15.9±2.0	16.0±1.6
	0.01	85.3±2.9	75.5±2.6	91.3±3.8	85.4±3.5	87.4±1.1
L-(+)-Glutamic Acid	0.05	74.4±3.8	77.1±4.1	20.0±2.0	77.5±4.3	23.2±2.0
	0.01	105.1±2.8	90.3±4.6	70.0±3.8	96.9±6.8	64.2±3.2
L-(+)-Lysine	0.1	19.3±1.2	57.5±2.3	20.4±3.6	25.9±2.6	28.3±2.3
	0.01	61.0±4.2	83.2±4.9	78.9±5.1	65.2±2.9	67.7±2.4

*Standard error of the mean.

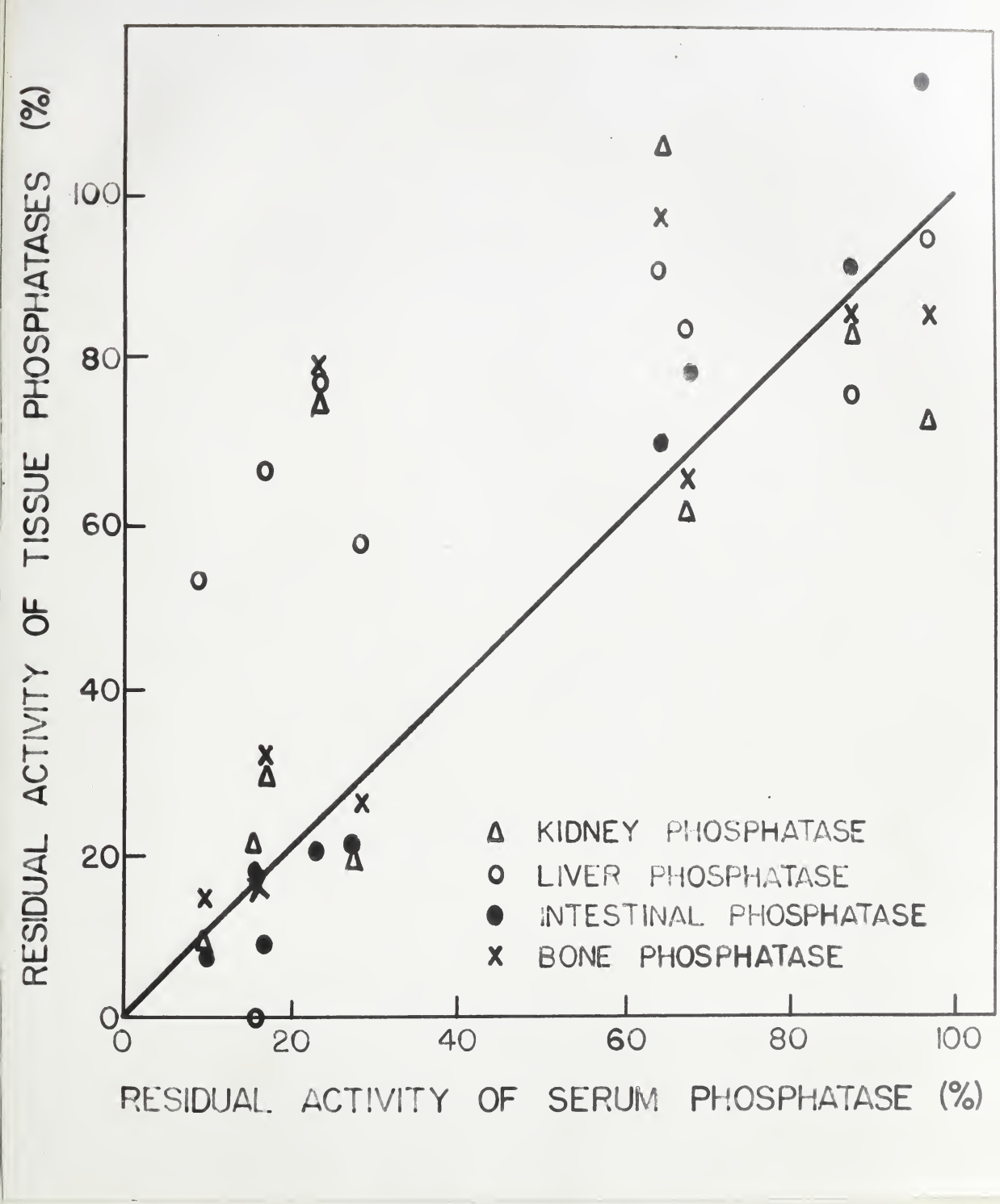

Fig. 4. Residual activity of tissue alkaline phosphatases in the presence of various inhibitors plotted against the corresponding residual phosphatase activity of serum in NORMAL adult rats. Each point represents the mean value with one inhibitor for 6 rats. See legend to Fig. 2.

TABLE IX

The Effect of Inhibitors on the Tissue Alkaline Phosphatase Activities of Starved Rats

Each value is the mean of 4 rats

Tissues		Kidney	Liver	Intestine	Bone	Serum
Original Values of Alkaline Phosphatase in Units/100 g. Wet Tissue: Serum, /100 ml.		4,670±170*	158±24	9,100±800	970±10	19±1.6
Inhibitor	**Molar Conc.**	**Residual Phosphatase Activities as Percentages of Original Values**				
Sodium Taurocholate	0.001	70.9±3.0*	97.3±6.0	112.8±4.8	84.0±1.2	95.5±2.6
Sodium Cyanide	0.01	13.0±0.9	59.5±6.3	7.0±0.6	13.6±1.7	7.9±1.4
	0.001	32.4±2.5	71.8±7.2	8.3±1.5	32.7±1.1	15.2±2.0
Sodium Oxalate	0.1	22.4±1.3	0	13.6±1.9	20.7±2.0	5.2±2.2
	0.01	88.5±5.9	75.5±2.5	87.3±2.9	89.5±6.6	89.7±0.8
L-(+)-Glutamic Acid	0.05	76.9±3.7	84.3±3.8	18.5±3.3	78.1±5.7	29.2±2.7
	0.01	105.4±3.6	108.3±6.3	60.8±5.0	109.0±8.0	66.1±2.9
L-(+)-Lysine	0.1	22.1±2.2	64.3±5.4	18.6±2.1	32.0±4.2	16.8±1.2
	0.01	63.1±4.6	87.8±6.8	70.9±4.4	71.6±3.9	68.5±3.6

*Standard error of the mean.

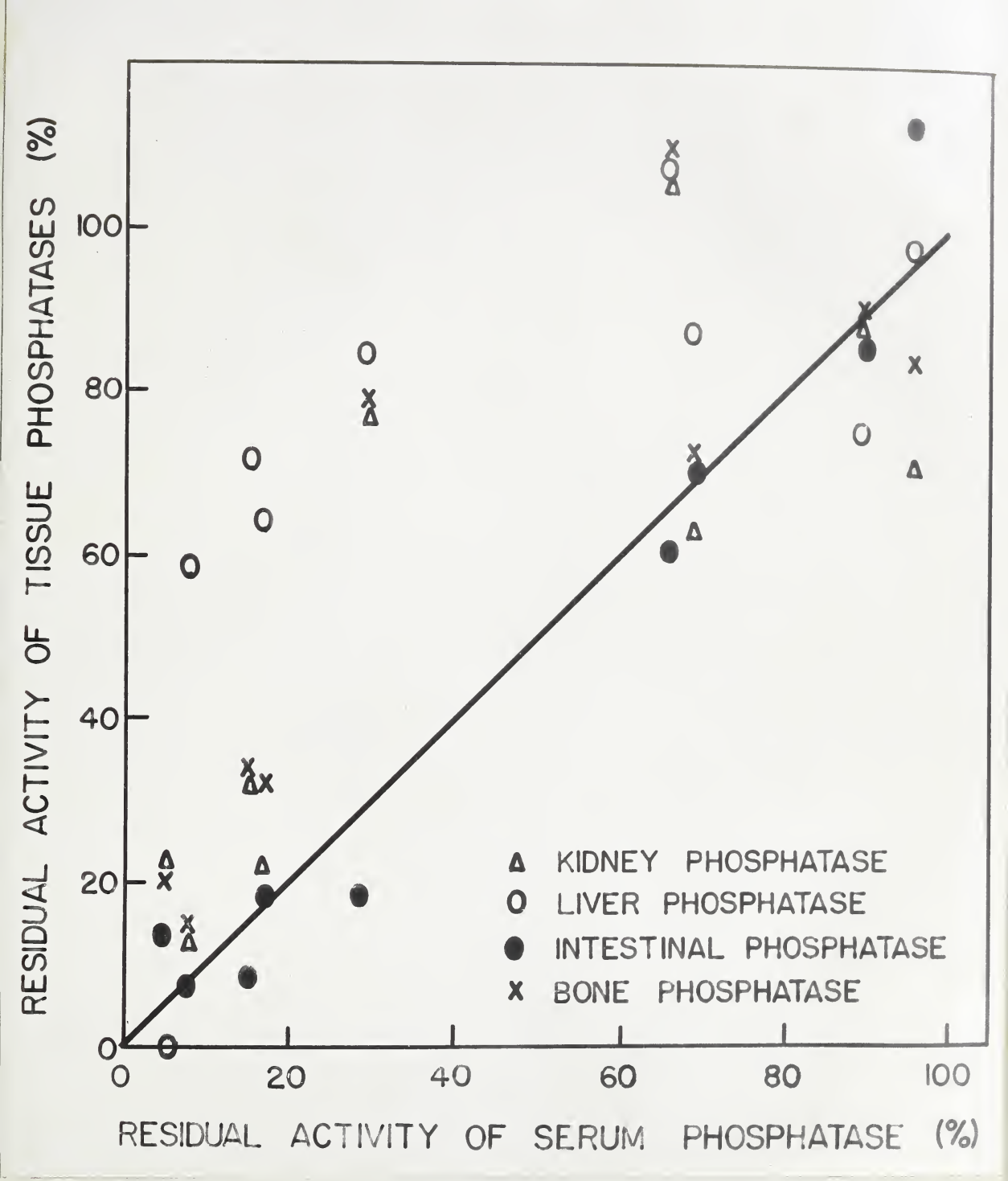

Fig. 5. Residual activity of tissue alkaline phosphatases in the presence of various inhibitors plotted against the corresponding residual phosphatase activity of serum in FASTING adult rats. Each point represents the mean value with one inhibitor for 4 rats. See legend to Fig. 2.

4. Discussion

The data in Tables VI and VII for the diabetic rats and those receiving a high fat diet show clearly that the inhibition pattern of serum phosphatase strikingly resembles that of the intestinal phosphatase, while the phosphatases of the other tissues manifest a distinctly different response to the inhibitors used. The similarity between serum and intestinal phosphatases is emphasized further by Figures 2 and 3. Therefore on the basis of the evidence presented here it may be said that the increase in serum alkaline phosphatase levels which is characteristic both of alloxan diabetes and of a high fat diet originates in the intestine. In support of this conclusion, it should be noted that the level of alkaline phosphatase in the intestine is much higher in the case of these two groups of animals than in the case of the normal or the starved rats.

The inhibition of the serum phosphatase of normal animals does not resemble that of the intestinal phosphatase as closely as in the preceding two groups of rats (Table VIII and Figure 4). However, the similarity is sufficient to justify the conclusion that the major portion of the serum alkaline phosphatase of normal rats is derived from the intestine, and this is

supported by the statements of other workers (27, 43). Normal rats have a much lower serum alkaline phosphatase level than either diabetic rats or those receiving a high fat diet, and so any contribution from tissues other than the intestine would be proportionally greater, and this would account for the lesser degree of similarity between intestinal and serum phosphatases which may be observed in Figure 4. In those rats which have abnormally high serum phosphatase levels the influence of contributions from other tissues would be virtually nullified by the overwhelming excess from the intestine.

The response of the serum alkaline phosphatase of starved rats deviates still more from the pattern for intestinal phosphatase (Table IX and Figure 5). This is to be expected, however, since the serum levels in fasting animals are only about 20% of the normal levels, and phosphatases from other tissues would contribute relatively more of the total activity. Bone is a probable source of some, if not all, of that portion of the serum phosphatase not supplied by the intestine. In the course of studies with rachitic rats, recently completed in this laboratory, it has been possible to demonstrate with the above inhibitors that the increased serum alkaline phosphatase levels associated

with this condition are, in all probability, derived from bone.

There is a significant correlation between the intestinal and serum phosphatase levels, when the four groups of animals are considered together. While the intestinal phosphatase activities of the normal animals appear to be lower than those of the starved rats, this may be accounted for by the fact that there was a 20% loss of weight by the intestine during the starvation period of one week.

With the exception of the liver, the effects of each inhibitor on the phosphatase activities of the tissues used above show little variation between the four groups of animals. The liver has the highest phosphatase activity in diabetes, and this is somewhat lower in the animals receiving the high fat diet, and still lower in the normal and in the starved rats. These changes approximately parallel the variations in levels of the serum enzyme. The inhibition of liver phosphatase by cyanide, glutamic acid and lysine becomes less as the liver enzyme level decreases, and the pattern of inhibition becomes increasingly different from that of the serum phosphatase. These observations support the view (4, 48, 56) that the liver has two alkaline phosphatases. One of these, the

cyanide insensitive "Fraction I" of Cloetens (4), is produced in the liver itself, and the other, the cyanide sensitive "Fraction II" of Cloetens, is a result of excretion by the liver of serum alkaline phosphatase into the bile. As the level of the cyanide sensitive serum phosphatase is decreased, from abnormally high to abnormally low values, the proportion and amount of the cyanide sensitive phosphatase in the liver decreases also.

5. Summary

The effect of five inhibitors on the alkaline phosphatases of the kidney, liver, intestine, bone and serum of rats in four different experimental states is presented and discussed. The phosphatases of the serum and the intestine of diabetic rats and of those receiving a high fat diet show similar reactions with the inhibitors: their patterns of behaviour are distinctly different from those of the other tissues. The evidence indicates that the increased serum alkaline phosphatase in these two experimental states originates in the intestine. In normal and in starved rats, on the other hand, the inhibition pattern of the serum enzyme does not resemble that of the intestinal enzyme as closely as in the preceding two experimental states. However, these effects are sufficiently alike to indicate that the major portion of the serum alkaline phosphatase is derived from the intestine. One or more of the other tissues may contribute a small portion to the total serum alkaline phosphatase.

Evidence is presented which supports the hypothesis that the liver has two alkaline phosphatases; one is produced in the liver itself and the other originates in the serum.

V. ON THE DIETARY COMPONENTS AFFECTING THE ALKALINE PHOSPHATASE IN RAT SERUM

1. Introduction

The previous sections have shown that the abnormally lowered but constant levels of serum alkaline phosphatase, which are characteristic of fasting rats, may originate in various tissues, including the intestine. However, the increased amounts of the serum enzyme in rats receiving a normal or high fat diet, as well as in alloxan diabetic animals, appear to be derived chiefly from the intestine. It has also been established that there is a highly significant correlation between serum alkaline phosphatase activity and the daily consumption of food, particularily fat (30). Since the increases in the serum enzyme over the low, basal fasting levels originate from the intestine, the problem of the functions of intestinal alkaline phosphatase might be approached by determining the dietary components which are responsible for the increased formation of the intestinal enzyme as reflected in the serum.

The earlier experiments of Weil and Russel (38) had shown that only the alcohol-ether soluble portion of Purina Dog Chow was effective in raising plasma alkaline phosphatase values of adult rats above the fasting level. They tested a large number of substances with respect

to their effect upon starvation phosphatase levels. Neither proteins nor carbohydrates had any effect, in contrast to Bodansky's conclusion (20) that only carbohydrates would restore the low fasting levels of young dogs to normal values. Glycerol, cholesterol, sodium B-glycerophosphate, dicarboxylic acids and saturated fatty acids had no effect. Only a few unsaturated fatty acids (oleic, erucic, linoleic and linolenic) were active in restoring fasting levels of serum phosphatase to normal. Elaidic acid, the geometrical isomer of oleic acid, was only slightly active, and a possible spatial effect was thus indicated. A free carboxyl group was found necessary for the effect on fasting levels, since oleic amide and oleyl alcohol were inactive. Cephalin restored fasting plasma phosphatase values to normal, whereas lecithin did not.

There is strong evidence (19, 57, 58) that absorbed fatty acids are changed into phospholipids within the intestinal mucosa and are transported in this form in the lymph and plasma (59, 60). Weil and Russel believed that the increased plasma alkaline phosphatase activity following the ingestion of unsaturated fatty acids might be linked with the process of transformation of phospholipids to neutral fat within various organs. They further felt that saturated fatty acids may have a

different metabolism since they do not affect plasma phosphatase levels. The latter suggestion has support in the work of Bloom, Chaikoff, Reinhardt and Dauben (61), who fed palmitic acid to rats and recovered 96% of it in the lymph as the free fatty acid. Thus it is possible that saturated fatty acids are not transformed into phospholipids during intestinal absorption, or at least are not transported as such.

Since it is now known that the increased serum alkaline phosphatase activity which results from the feeding of fats is probably derived from the intestine, it seems probable that phosphatase functions in the absorption of some fatty acids from the intestine. It was decided to reinvestigate the problem with larger groups of animals, and to feed them a basal fat free diet supplemented with various derived lipids and fat soluble vitamins for several weeks.

2. Experimental

Adult male albino rats weighing at least 250 grams were placed in individual cages and given food and water ad libitum. Serum alkaline phosphatases, daily food consumptions and body weights were measured once per week. The rats were given ground Purina Fox Checkers for one week before taking the "0 Values". Groups of six rats each were then placed on the diets listed below for 4 weeks. All groups were then starved for 1 week.

Diet 1 was the basal fat free diet consisting of 20% vitamin free casein (Smaco), 76% sucrose, 4% McCollum's Salt Mixture, together with adequate amounts of the B-group vitamins; and the fat soluble vitamins in crystalline form at the rate of 1.3 mg. vitamin A acetate, 0.15 mg. calciferol and 133 mg. α-tocopherol, per kilo of diet. Diet 2 consisted of the basal diet minus the fat soluble vitamins. Diets 1 and 2 would thus test the effect on serum phosphatase of the fat soluble vitamins A, D and E, and the non-fat portions of the diet. The remaining diets were devised to test the effect on the enzyme of lipid derivatives equivalent to the concentrations which would be present in a diet containing 10% triglyceride. The lipid derivatives were added to the basal diet and replaced an equal amount of sucrose.

Diet 3 contained 1.04% glycerol; diet 4, 9.57% stearic acid; and diet 5, 9.57% oleic acid. Diet 6, which served as a control, consisted of the basal diet with 10% hydrogenated vegetable oils (Crisco) replacing an equal amount of sucrose.

3. Results

The results are indicated in Tables X to XV. Figure 6 illustrates the fluctuations in the phosphatase levels of the six groups during the experiment.

TABLE X

FAT SOLUBLE VITAMINS

The effect of the basal fat free diet (diet 1) on Serum Alkaline Phosphatase (units/100 ml.) (P); Daily Food Consumption (grams) (C) and Body Weights (grams) (W) of adult rats. (Means of six animals).

Time	P	C	W
0 Values	74 ± 5*	13.5 ± 1.0	280 ± 9
1 W 1 Week	23 ± 2	13.8 ± 0.8	289 ± 26
2 Weeks	28 ± 2	11.2 ± 0.8	306 ± 11
3 Weeks	32 ± 3	14.7 ± 0.5	319 ± 12
4 Weeks	27 ± 2	15.7 ± 0.6	333 ± 10
Starved one week	27 ± 2		264 ± 11

*Standard error of the mean.

TABLE XI

FAT FREE DIET

The effect of the basal fat free diet minus the fat soluble vitamins A, D and E (diet 2) on Serum Alkaline Phosphatase (units/100 ml.) (P); Daily Food Consumption (grams) (C) and Body Weights (grams) (W) of adult rats. (Means of six animals).

Time	P	C	W
0 Values	88 ± 8*	14.3 ± 0.7	285 ± 11
1 Week	26 ± 2	13.0 ± 1.4	281 ± 17
2 Weeks	30 ± 1	12.2 ± 0.5	295 ± 16
3 Weeks	31 ± 1	14.5 ± 0.7	311 ± 16
4 Weeks	26 ± 2	16.5 ± 0.6	323 ± 15
Starved one week	33 ± 3		258 ± 14

*Standard error of the mean.

TABLE XII

GLYCEROL

The effect of the basal fat free diet plus Glycerol (diet 3) on Serum Alkaline Phosphatase (units/100 ml.) (P); Daily Food Consumption (grams) (C) and Body Weights (grams) (W) of adult rats. (Means of six animals).

Time	P	C	W
0 Values	90 ± 3*	16.2 ± 0.6	310 ± 13
1 Week	27 ± 2	16.2 ± 0.6	310 ± 12
2 Weeks	24 ± 2	13.0 ± 0.5	322 ± 12
3 Weeks	29 ± 1	16.5 ± 0.8	336 ± 11
4 Weeks	26 ± 2	16.3 ± 0.8	347 ± 10
Starved one week	29 ± 3		282 ± 9

*Standard error of the mean.

TABLE XIII

STEARIC ACID

The effect of the basal fat free diet plus Stearic Acid (diet 4) on Serum Alkaline Phosphatase (units/ 100 ml.) (P); Daily Food Consumption (grams) (C) and Body Weights (grams) (W) of adult rats. (Means of six animals).

Time	P	C	W
0 Values	80 ± 6*	13.8 ± 0.9	279 ± 7
1 Week	32 ± 2	12.5 ± 0.8	284 ± 6
2 Weeks	42 ± 3	13.3 ± 0.8	295 ± 6
3 Weeks	53 ± 5	13.5 ± 1.0	302 ± 7
4 Weeks	44 ± 4	11.7 ± 0.6	309 ± 6
Starved one week	33 ± 4		248 ± 5

*Standard error of the mean.

TABLE XIV

OLEIC ACID

The effect of the basal fat free diet plus Oleic Acid (diet 5) on Serum Alkaline Phosphatase (units/100 ml.) (P); Daily Food Consumption (grams) (C) and Body Weights (grams) (W) of adult rats. (Means of six animals).

Time	P	C	W
0 Values	77 ± 14*	12.7 ± 1.2	284 ± 7
1 Week	75 ± 5	12.0 ± 0.6	291 ± 9
2 Weeks	78 ± 2	15.8 ± 0.3	309 ± 9
3 Weeks	89 ± 3	13.8 ± 0.9	320 ± 10
4 Weeks	80 ± 4	13.0 ± 0.6	334 ± 10
Starved one week	31 ± 2		267 ± 9

*Standard error of the mean.

TABLE XV

HYDROGENATED VEGETABLE OILS

The effect of the basal fat free diet plus hydrogenated vegetable oils (Crisco) (diet 6) on Serum Alkaline Phosphatase (units/100 ml.) (P); Daily Food Consumption (grams) (C) and Body Weights (grams) (W) of adult rats. (Means of six animals).

Time	P	C	W
0 Values	90 ± 5*	16.5 ± 1.4	297 ± 12
1 Week	70 ± 5	13.8 ± 0.6	306 ± 9
2 Weeks	71 ± 5	13.2 ± 0.4	320 ± 8
3 Weeks	82 ± 4	13.7 ± 0.7	331 ± 9
4 Weeks	76 ± 4	13.5 ± 0.6	343 ± 8
Starved one week	33 ± 1		279 ± 8

*Standard error of the mean.

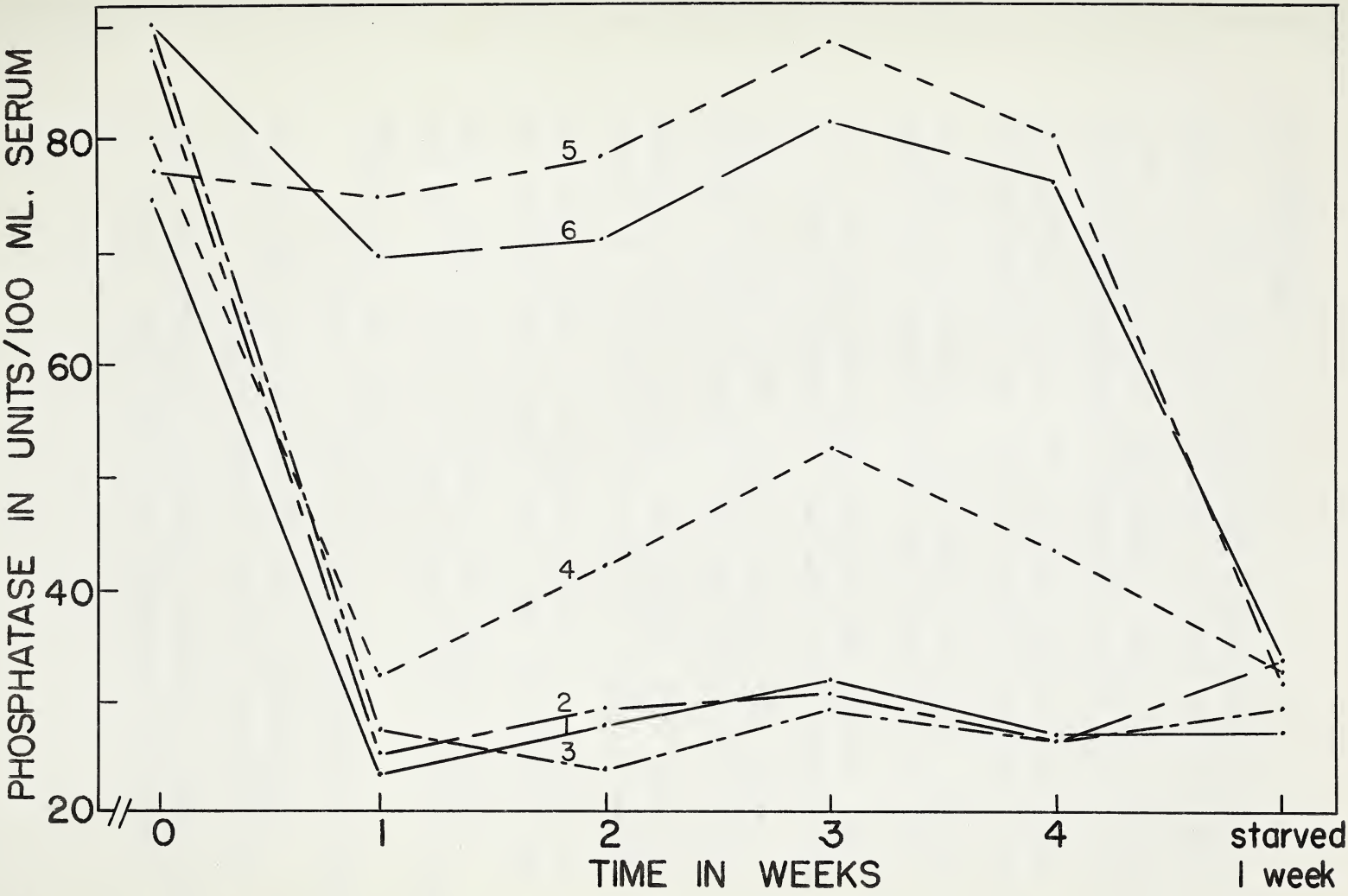

Fig. 1. The mean serum alkaline phosphatase levels of groups of rats as a result of (a) a pre-experimental diet of ground Checkers for 1 week, (b) test diets 1-6 for 4 weeks, and (c) post-dietary starvation for 1 week.

4. Discussion

There are no significant differences in food consumption on the basis of which the serum alkaline phosphatase levels may be interpeted. There is no difference, statistically speaking, between the weight gains of the six groups of animals for the four weeks on the diets, despite the fact that diets 1, 2 and 3 contained no fat and diet 2 contained no fat soluble vitamins. This is taken as an indication that food consumption was adequate and that the animals were in good health. This is a little surprising in the case of the group on the diet deficient in fat soluble vitamins. It serves, however, as an indication that the animals had a good reserve of these vitamins and therefore that only the effects of depleting the diet of fat soluble vitamins was being tested. No attempt was made to produce vitamin deficiencies in these rats. The observations made above regarding the absence of fat soluble vitamins from diet 2 would also apply to the absence of essential fatty acids from diets 1 to 5.

An analysis of variance of the phosphatase levels for the four weeks on the test diets indicates that there is a highly significant variation between some diets. There is, however, no significant variation between diets 1, 2 and 3. Since there is no difference between the

serum phosphatase values of rats on these diets and the same rats after one week of post-dietary starvation (Figure 6), it may be said that the fat soluble vitamins A, D and E, glycerol, and the non-fat portions of the diet do not affect the fasting levels of serum alkaline phosphatase. This result agrees with that of Weil and Russel (38), who did not, however, test the effect of the fat soluble vitamins on fasting serum alkaline phosphatase levels.

There is no significant variation between the serum phosphatase levels of the six groups of animals after one week of post-dietary starvation. Diets 4, 5 and 6 produced levels of serum phosphatase which were significantly greater than these starvation levels or the levels on the first three diets. Stearic acid (diet 4) produced an activity greater than the activity of the enzyme during starvation, although not as great as the activity produced by oleic acid (diet 5). The effect of stearic acid, however, did not show up immediately, and the phosphatase level after one week on diet 4 was the same as the starvation level (Table XIII). This may explain why Weil and Russel (38) did not observe any effect from this fatty acid in their short term experiments. It would appear from these results that alkaline phosphatase may be involved in

the intestinal absorption of stearic and possibly other saturated fatty acids as well as the unsaturated fatty acids.

Both oleic acid and Crisco produced serum alkaline phosphatase levels comparable to the normal values on Checkers (Table XIV and XV and Figure 6). Oleic acid produced a serum phosphatase activity that was slightly but significantly greater than the activity on the Crisco diet. These results agree with those of Weil and Russel (38).

5. Summary

Adult rats maintained for 4 weeks on fat free diets with or without the fat soluble vitamins A, D and E showed a gain of weight equivalent to that of rats on diets containing fats and fat soluble vitamins. The fat soluble vitamins, glycerol, and the non-fat portions of the diet do not produce serum alkaline phosphatase levels higher than those found in starvation states. Stearic acid produces serum alkaline phosphatase levels higher than those resulting from starvation, although not as high as the levels produced by oleic acid or "Crisco". Thus alkaline phosphatase may be involved in the intestinal absorption of saturated fatty acids as well as unsaturated fatty acids.

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